C_{26} Sterol in a Human Urine

Abstract. *A new C_{26} sterol, 22-*trans*-27-norcholesta-5,22-dien-3β-ol, was found in the urine of a 6-year-old girl, with a clinical diagnosis of congenital adrenal hyperplasia of the salt losing type, accompanied by symptoms of mixed sex anatomy and skin pigmentation. The structure of the sterol was determined by comparison with the synthetic compound. The sterol was also detected in the serum. This appears to be the first case in which a C_{26} sterol has occurred in mammalia.*

During our studies on the analysis of urinary steroids, we found a C_{26} sterol, 22-*trans*-27-norcholesta-5,22-dien-3β-ol (**1**), as a major steroid in the urine of a 6-year-old girl, clinically diagnosed as having congenital adrenal hyperplasia.

The patient (N.K.) was born 22 January 1971 (birth weight, 2.7 kg); ambiguous genitalia (clitoral enlargement, labioscrotal swelling with heavy pigmentation, and opening of only the urogenital sinus) and generalized skin pigmentation were observed at birth. No history of consanguinity, nor of another similar individual in the family is known. The mother was healthy and had no medication during the pregnancy. The patient was admitted at the age of 11 days to the Kanagawa Children's Medical Center for sex determination.

The buccal smear was chromatin positive and the karyotype of lymphocytes was 46,XX. Low serum sodium (125 to 128 meq/liter), low chloride (98 to 102 meq/liter), high potassium (7.7 to 10.1 meq/liter), and metabolic acidosis (*p*H, 7.330, base excess −13 meq/liter) were observed. The urinary 17-ketogenic steroid (*1*) was high (6.6 mg/day), 17-ketosteroids (0.75 to 1.1 mg/day) and the ratio of 11-deoxyketogenic steroids to 11-oxyketogenic steroids (0.57) were also slightly high; pregnanediol (0.01 mg/day) and tetrahydrodesoxycortisol (undetectable) were normal, and 17-hydroxycorticosteroid was undetectably low. These observations suggest failure of 21-steroid hydroxylase. But urinary pregnanetriol (0.01 mg/day) was not elevated. However, as a similar finding was reported (2) in a neonatal 21-steroid hydroxylase deficiency, the possibility of another enzyme defect, such as 3β-hydroxysteroid dehydrogenase, remained.

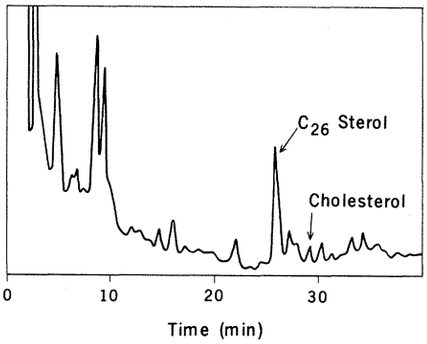
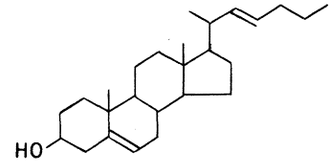


Fig. 1. Gas chromatogram of urinary steroids analysis. Urine (50 ml) was incubated with β-glucuronidase at 37°C and *p*H 6.0 for 24 hours, dissolved with a mixture of 50 percent H_2SO_4 and ethyl acetate at 34°C for 48 hours, and then extracted with ethyl acetate. The extract was treated with methoxyamine in pyridine and then trimethylsilylimidazole. A portion was treated by gas chromatography, 3 percent OV-1 on Shimalite W (80 to 100 mesh); the column was 150 cm by 4 mm (inside diameter), programmed at 180° to 280°C.

After the patient was treated with hydrocortisone and fluorohydrocortisone, serum electrolytes and urinary steroids became normal, and the skin pigmentation disappeared. When the patient was 6 years old, the oral maintenance dose of hydrocortisone (25 mg/day) was replaced by dexamethasone (0.7 mg daily) and synthetic adrenocorticotropic hormone (ACTH; tetracosactide zinc hydroxide) was injected (20 international units daily) for 3 days. Estimation by conventional clinical methods showed that the amounts of urinary 17-ketosteroids, 17-ketogenic steroids, and pregnanetriol and serum testosterone, dehydroepiandrosterone, and progesterone were almost unchanged by the injection, and serum 17-hydroxyprogesterone and dehydroepiandrosterone sulfate were slightly increased from 20 ng/dl to 95.5 ng/dl and from 11.8 μg/dl to 23.6 μg/dl, respectively. Urine and serum samples were then analyzed by gas chromatography-mass spectrometry (GC/MS) before and after the ACTH treatment.

The urine for analysis was treated as described (*3*). After hydrolysis of the treated urine with β-glucuronidase and solvolysis with sulfuric acid, the steroids were extracted with ethyl acetate and treated to form the methyloxime-trimethylsilyl ether derivative. Analysis by gas liquid chromatography (GLC) of the mixture revealed a major peak with a retention time of 26.3 minutes (Fig. 1). The mass spectrum of the compound obtained by GC/MS analysis showed prominent peaks at *m/e* 442 (27 percent), 427(9), 352(44), 337(21), 313(56), 255(35), 129(83), and 97(100), an indication of the presence of the trimethylsilyl (TMS) ether of a C_{26} sterol. The spectrum was quite similar to that of the TMS derivative of 22-*trans*-24-norcholesta-5,22-dien-3β-ol (**2**) (*4*), but the GLC retention time of the metabolite was a little longer than that of compound **2** (*5*). Authentic samples of 27-norcholesta-5,22-dien-3β-ol (**1**) and 24-norcholesta-3,23-dien-3β-ol (**3**) were then synthesized for comparison. Compound **1** was prepared by the method of Djerassi (*6*) from the C_{22} aldehyde by the Wittig reaction with *n*-butyltriphenylphosphonium bromide. The resulting 22-*cis* and -*trans* isomers were separated by thin layer chromatography on silver nitrate impregnated silica gel. Compound **3** was obtained by the Grignard reaction of the C_{23} aldehyde with isopropyl magnesium bromide followed by phosphorus pentoxide dehydration and alkaline hydrolysis of the acetyl group. The mass spectrum of the compound **1** was identical with that of the unknown sterol (*7*). The GLC retention time of the unknown sterol on a glass capillary column was also identical with that of the *trans* isomer of compound **1** (*8*). Thus, the unknown sterol was unambiguously



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identified as 22-*trans*-27-norcholesta-5,22-dien-3β-ol (**1**). The amount in urine was calculated to be about 50 μg/100 ml. For detection of the same sterol in serum, the unsaponifiable fraction was analyzed. The serum (0.5 ml) was treated with 5 ml of KOH in methanol (5 percent) for 1 hour at 60°C. After addition of water, the mixture was centrifuged, and the supernatant was extracted with

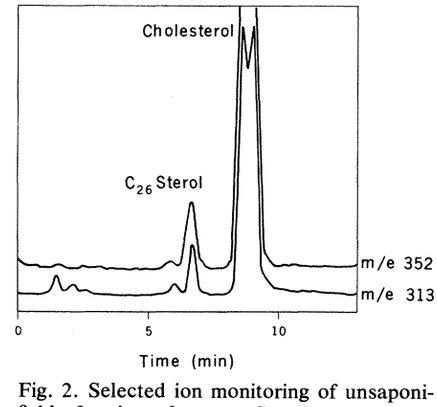

Fig. 2. Selected ion monitoring of unsaponifiable fraction of serum. Serum was saponified with KOH-methanol; the unsaponifiable fraction was dissolved in trimethylsilylimidazole (40 μg), and 2 μl was injected to GC/MS system. The fragment ions *m/e* 352($M^+ - 90$) and 313($M^+ - 129$) were selected.

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ether. The GLC analysis of the ether extract showed a strong peak for cholesterol, but no peak corresponding to the C_{26} sterol could be detected. However, the compound could be clearly detected by GC/MS analysis (*9*) of the sample by means of selected ion detection technique and by monitoring the peaks at *m/e* 352 and 313 (Fig. 2). The estimated amount was about 40 ng/ml.

The C_{26} sterol (compound **2**) was first isolated by Idler *et al.* (*10*) from the scallop *Placopecten magellanicus*, and since then several 24-norcholesterol derivatives were identified in a number of marine sources, such as asteroid ($\Delta^{7,22}$) (*11*), clam ($\Delta^{5,22}$) (*12*), jellyfish (Δ^{22} and $\Delta^{5,22}$) (*13*), *Tunicier halocynthia roretzi* (Δ^{22}, $\Delta^{5,22}$, and $\Delta^{7,22}$) (*14*), and red algae (Δ^{22}, $\Delta^{5,22}$) (*15*). Two 27-norsterols, 22-*trans*-27-nor-24-methylcholesta-5,22-dien-3β-ol and its Δ^7-isomer, were isolated from the asteroid *Asterias amurensis* (*16*). But those are all from marine sources and are minor components of the sterol fraction.

This appears to be the first case of detection of a C_{26} sterol in mammalia. At present, we have no information on the source or the biosynthesis of this sterol. In view of the characteristic symptoms, the C_{26} sterol (produced by an abnormal metabolic pathway) may not be a suitable substrate of cholesterol $C_{20,22}$ lyase (cytochrome P 450), which is a key enzyme for steroid hormone biosynthesis. Alternatively, the function of cholesterol $C_{20,22}$ lyase might be disrupted for some reason, and the degradation of cholesterol side chain may be directed instead toward production of the C_{26} sterol.

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References and Notes

1. Drektor's method was used for 17-ketogenic steroid determination, Few's method for 17-ketosteroids, Stern's method for pregnanetriol, and Klopper's method for pregnanediol.
2. C. H. Shackleton, F. L. Mitchell, J. W. Farquhar, *Pediatrics* **49**, 198 (1972).
3. E. C. Horning *et al.*, *J. Gas Chromatogr.* (1967), p. 283.
4. The sample isolated from the clam *Tapes philippinarum* was provided by Dr. A. Kanazawa, University of Kagoshima.
5. Retention times of GLC with the use of 1.5 percent OV-17 on Shimalite W (80 to 100 mesh), a column 150 cm by 4 mm (inside diameter), at 255°C, were as follows: 22-*trans*-27-norcholesta-5,22-dien-3β-ol (**1**), 6.5 minutes; 22-*cis*-27-norcholesta-5,22-dien-3β-ol, 6.4 minutes; 24-norcholesta-5,22-dien-3β-ol (**2**), 5.3 minutes, 24-norcholesta-3,23-dien-3β-ol (**3**), 7.6 minutes. GLC analysis of urinary steroids of a normal girl shows a similar profile as reported by Horning, *et al.* [E. C. Horning and M. G. Horning, *J. Chromatogr. Sci.* **9**, 129 (1971); A. L. German and E. C. Horning, *ibid.* **11**, 76 (1973)], indicating many small peaks of steroids in the region of retention time of 15 to 40 minutes on the analytical conditions of Fig. 1. The urine from the lipoid hyperplasia which is one of the congenital adrenal hyperplasia showed a major peak of cholesterol without C_{26} sterol in the GLC analysis.
6. Y. M. Sheikh and C. Djerassi, *Steroids* **26**, 129 (1975).
7. Mass spectrum of compound **2** showed *m/e* 442 (13 percent), 427(4), 352(23), 337(9), 313(22), 255(25), 129(45), and 97(100); that of compound **3** showed *m/e* 442 (14 percent), 427(12), 352(24), 343(48), 337(15), 313(34), 283(100), 253(20), and 129(68).
8. *Cis*- and *trans*-27-norcholesta-5,22-dien-3β-ols were separated by a glass capillary column with Poly-I 101, 30 m by 0.2 mm (inside diameter) at 245°C. The retention time for the *trans* isomer was 10.7 minutes, and for the *cis* isomer it was 10.3 minutes.
9. A Shimadzu-LKB-9000 GC-MS instrument with MID-PM was used for multi-ion monitoring; column, 1.5 percent OV-17 on Shimalite W(80–100 mesh), 150 cm by 4 mm (inside diameter) at 250°C.
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16. M. Kobayashi and H. Mitsuhashi, *Tetrahedron* **30**, 2147 (1974).
17. This is part 55 in the series of "Studies on Steroids." Part 54, see M. Yoshida, K. Kuretani, M. Ishiguro, M. Ikekawa, *J. Pharmacol. Dyn.* **2**, 92 (1979).

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Non-Inactivation of an X-Chromosome Locus in Man

Abstract. *Cloned fibroblasts from women heterozygous for X-linked ichthyosis (steroid sulfatase deficiency) were examined to see whether or not this locus is subject to X-inactivation. Of 103 clones examined, all had normal levels of steroid sulfatase activity. Two of the women studied were also heterozygous for glucose-6-phosphate dehydrogenase deficiency. This allowed the demonstration that both X chromosomes were represented as the active X in various clones and that selection did not account for these findings. Thus, the steroid sulfatase locus, like the* Xg^a *locus to which it is linked, appears to escape X-inactivation in man.*

One of the two X chromosomes in normal female somatic cells is functionally inactivated at an early stage of embryogenesis in all mammals (*1*). This inactivation process teleologically serves the function of achieving "dosage compensation" for X-chromosome loci between males and females (*2*). In eutherian mammals, either the maternally or paternally derived X chromosome is inactivated in a random fashion and the pattern of X-inactivation, once established, is genetically fixed for any given cell and its progeny. Much support for this theory, most clearly espoused by Lyon (*3*), has been obtained in many species, including man. Davidson *et al.* (*4*) used cloned fibroblasts from females, heterozygous for an X-linked biochemical marker [an electrophoretic variant of glucose-6-phosphate dehydrogenase (G6PD : E.C. 1.1.1.49)], to demonstrate

Table 1. Steroid sulfatase activity in cultured fibroblasts from normal individuals, patients with X-linked ichthyosis (including the sons of four obligate heterozygotes A, B, C, and D), heterozygotes for X-linked ichthyosis, and clones derived from these heterozygotes.

Fibroblasts	*N*	Cholesterol sulfatase (pmole/mg protein-hour)	
		Mean	Range
Control lines	18	4.38	1.10 to 9.20
Patients with X-linked ichthyosis	30	< 0.15	0 to 0.15
Heterozygote A		3.13	
Heterozygote B		1.59	
Heterozygote C		6.31	
Heterozygote D		3.47	
Heterozygote A clones	13	2.79	1.15 to 5.26
Heterozygote B clones	19	2.71	1.42 to 5.13
Heterozygote C clones	39	4.77	0.96 to 10.45
Heterozygote D clones	32	3.85	1.80 to 6.33
Son of heterozygote A (K III-8)		< 0.10	
Son of heterozygote B (F IV-2)		< 0.10	
Son of heterozygote C (C III-8)		< 0.10	
Son of heterozygote D (D III-3)		< 0.10	

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